OXFORD Toxicology Research

Toxicology Research, 2022, **11**, 644–653
https://doi.org/10.1093/toxres/tfac039
Advance access publication date: 8 July 2022
Paper

The designer benzodiazepine, flubromazepam, induces reward-enhancing and cardiotoxic effects in rodents

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The use of many benzodiazepines is controlled worldwide due to their high likelihood of abuse and potential adverse effects. Flubromazepam—a designer benzodiazepine—is a long-acting gamma-aminobutyric acid subtype A receptor agonist. There is currently a lack of scientific evidence regarding the potential for flubromazepam dependence or other adverse effects. This study aimed to evaluate the dependence potential, and cardiotoxicity via confirmation of the QT and RR intervals which are the factors on the electrical properties of the heart of flubromazepam in rodents. Using a conditioned place preference test, we discovered that mice treated intraperitoneally with flubromazepam (0.1 mg/kg) exhibited a significant preference for the flubromazepam-paired compartment, suggesting a potential for flubromazepam dependence. In addition, we observed several cardiotoxic effects of flubromazepam; 100-μM flubromazepam reduced cell viability, increased RR intervals but not QT intervals in the electrocardiography measurements, and considerably inhibited potassium channels in a human ether-à-go-go-related gene assay. Collectively, these findings suggest that flubromazepam may have adverse effects on psychological and cardiovascular health, laying the foundation for further efforts to list flubromazepam as a controlled substance at both national and international levels.

Key words: cardiotoxicity; conditioned place preference (CPP); dependence potential; electrocardiography (ECG); flubromazepam; gamma-aminobutyric acid subtype A (GABAA); human ether-a-go-go-related gene (hERG); self-administration (SA).

Introduction

New psychoactive substances (NPSs) have been increasing in both quantity and availability at an alarming rate since their emergence.[1,2] NPSs are defined as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat."[1] According to the United Nations Office of Drug Crime, >800 NPSs had been reported as of December 2017.[3] Benzodiazepines are depressants that are widely prescribed to treat anxiety, insomnia, muscle spasms, alcohol withdrawal, and epilepsy, and the number of benzodiazepine analogs has increased in recent years.[4,5] Benzodiazepine NPSs, also known as "designer benzodiazepines," are not used for legal purposes[6]; at least 24 of these compounds have been identified as of November 2018.[7] Designer benzodiazepines are sold by online marketplaces at low prices without restrictions, thus posing a threat to public health worldwide.[8–10] Monitoring the availability and use of these compounds is challenging as they are synthesized via only slight changes to the chemical structures of existing benzodiazepines. Furthermore, there is currently scarce scientific evidence of their ill effects, including their dependence potential.

Recreational use of designer benzodiazepines poses a greater risk than medicinal use, as various doses can be used without proper knowledge of the pharmacological and toxicological effects.[4] These concerns have been realized, as evidenced by the increasing number of fatal and nonfatal intoxication cases involving designer benzodiazepines. The most frequently reported adverse effects include central nervous system depression with a sedative-hypnotic toxidrome, slurred speech, tachycardia, and impaired driving.[11]

Flubromazepam is classified as a designer benzodiazepine, along with phenazepam, etizolam, and diclazepam.[11] Flubromazepam was first synthesized in the early 1960s[12] and was recognized in 2013 as a recreational drug.[13] It is a controlled substance in

Received: January 28, 2022. **Revised:** May 10, 2022. **Accepted:** June 10, 2022

the United States of America (Schedule I), the United Kingdom (Class C), Germany (Anlage II), and the Republic of Korea (Schedule I psychoactive substance). Similar to other benzodiazepines, the major pharmacological target of flubromazepam is the gamma (γ)-aminobutyric acid type A (GABAA) receptor located in postsynaptic membranes in the brain.[8] Benzodiazepines act as positive allosteric modulators of the GABAA receptor by binding to a specific high-affinity binding site. The psychological effects of benzodiazepines, including sedation, anterograde amnesia, anticonvulsion, and dependence, are mediated by the α1-subunit of GABAA.[11]

More recently, a pharmacokinetic study performed in humans reported a terminal elimination half-life of flubromazepam of 10–20 h,[14] whereas previous studies had reported an elimination half-life of 106 h.[8,12,13] Because of its considerably long half-life, flubromazepam poses additional risks, such as overdose,[9] especially during withdrawal management. According to a previous report on acute intoxication cases presenting for emergency care in Sweden during 2012–2016, flubromazepam was the third most prevalent compound out of 14 designer benzodiazepines.[5,11,15] Flubromazepam has been involved in several lethal and nonlethal intoxication cases, mostly when mixed with other substances, such as synthetic opioids.[4,7,16] Adverse symptoms associated with flubromazepam use include coma, hypotension, and rhabdomyolysis[17]; however, because of the lack of scientific evidence of its pharmacological properties, its effects can only be deduced through anecdotal reports. This has made it very challenging to legally control the availability and use of flubromazepam, demonstrating an important need for scientific data on its dependence potential and toxic effects.

The present study aimed to examine the dependence liability and cardiotoxicity of flubromazepam. Dependence liability was investigated using behavioral models based on conditioned place preference (CPP) and self-administration (SA) behavioral paradigms.[18,19] As adverse effects on the cardiovascular system can be life-threatening, cardiotoxicity of flubromazepam was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, electrocardiography (ECG), human ether-à-go-go-related gene (hERG) assay, and measuring p21-activated kinase 1 (PAK1) protein levels.

Materials and methods

Animals

Male C57BL/6J mice (age: 6–8 weeks; weight: 25–30 g), male Sprague Dawley (SD) rats (age: 6–7 weeks; weight: 280–300 g) for ECG, and timed-pregnant SD rats for primary cardiomyocyte culture were provided by the National Institute of Food and Drug Safety Evaluation (Chungju, Korea), a member of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, Osong, Korea). The animals were maintained in a humidity- (55 ± 5%) and temperature-controlled (23 ± 1°C) environment with a 12 h/12 h light–dark cycle (lights on from 07:00 to 19:00 h). Laboratory mouse chow and water were provided ad libitum. Animals were acclimated for 1-week prior to the start of experiments. Handling occurred only during light cycles. All animal experiments in the present study were approved by the National Institute of Food and Drug Safety Evaluation/Ministry of Food and Drug Safety Animal Ethics Board (approval number: MFDS-18-018, MFDS-18-019).

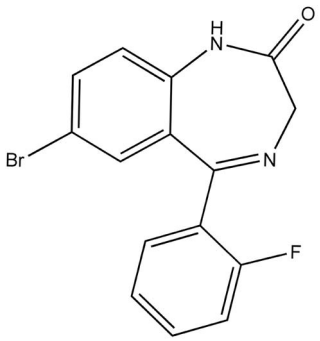

Fig. 1. Chemical structure of flubromazepam.

Chemicals and cell lines

Flubromazepam (purity >98%, Fig. 1) obtained from Kyung Hee University (Seoul, Korea) and methamphetamine hydrochloride (purity >98%) purchased from Samung Industry (Seoul, Korea) were dissolved in the vehicle solution (saline:dimethyl sulfoxide (DMSO):Tween 80 = 18:1:1). The H9c2 cell line (CRL-1446) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). Heparin was purchased from JW Pharma Co., Ltd (Seoul, Korea). DMSO and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, United States).

Apparatus

The CPP apparatus was obtained from Med Associates Inc. (St. Albans, VT, United States) and consisted of 3 compartments (black, gray, and white) that were separated by guillotine doors. The sizes of the white and black compartments were 13.5 × 17.5 × 15.5 cm, while the middle gray compartment (start box) was 13.5 × 9.5 × 15.5 cm. The animals were initially placed in the middle gray compartment before scoring the time spent in either the black or white compartment (pretest and posttest). The compartments were illuminated by dim light (12 lx). The time spent in each compartment was recorded using an infrared sensor controller system (Med Associates Inc., St. Albans, VT, United States). The SA test apparatus was also obtained from Med Associates Inc. (St. Albans, VT, United States), and the dimensions of the operant chamber were 24.1 × 20.3 × 18.4 cm. The chamber contained 2 levers: an active lever connected to an infusion pump and an inactive lever. The infusion pump was connected

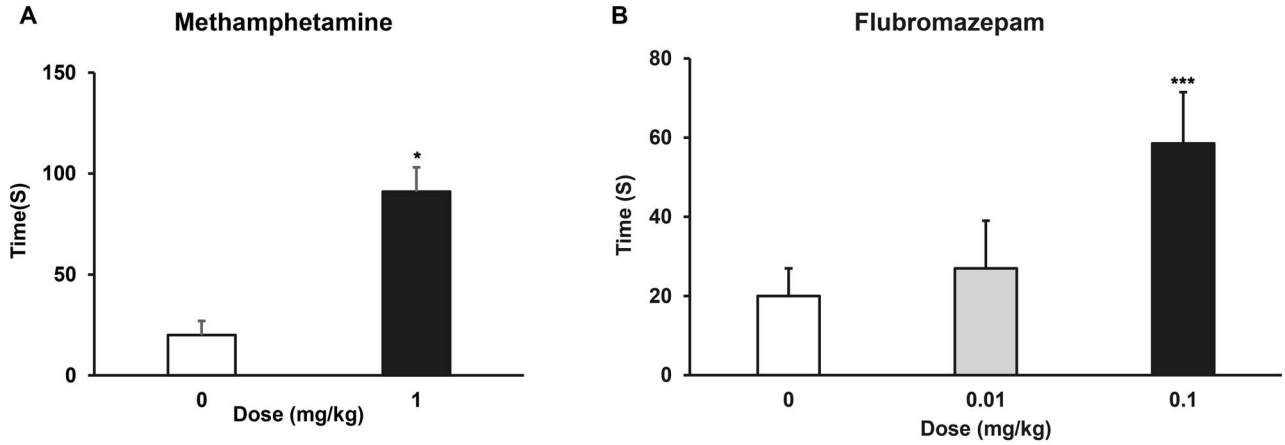

Fig. 2. Place preference induced by A) methamphetamine and B) flubromazepam. Data are expressed as mean ± standard error of the mean of 8 animals per group. *$P < 0.05$ and ***$P < 0.001$ indicate statistical significance compared with the vehicle control-treated group as determined by 1-way ANOVA followed by Tukey's multiple comparison test.

to a 1-mL syringe placed outside the operant chamber. A computerized system (schedule manager) connected to the SA test apparatus was used to record the data (Med Associates Inc., Fairfax, VT, United States).

CPP test

The CPP test consisted of 4 phases: (i) habituation, (ii) preconditioning, (iii) conditioning, and (iv) post-conditioning. (i) Habituation—on days 1–3, the mice were placed in the middle gray compartment and allowed access to both compartments of the apparatus for 30 min, once per day. (ii) Preconditioning—on day 4, the mice were allowed access to both compartments for 15 min without treatment. The time spent in each compartment was recorded, and these values were used as the baseline. Mice that did not show a preference for any particular compartment were selected and divided into 6 groups. (iii) Conditioning—from days 5–14, the mice were injected intraperitoneally (i.p.) with either the vehicle or flubromazepam (0.01 mg/kg or 0.1 mg/kg) before being placed in the white compartment for 40 min with the guillotine door closed. The next day, they received the vehicle and were placed in the black compartment for 40 min. This process was repeated 5 times over 10 days. And, (iv) Post-conditioning—on day 15, the mice were allowed access to the white and black compartments for 15 min. The time spent in each compartment was measured and used as the test value. The outcomes were calculated based on the difference between post-conditions (test values) and preconditions (baseline values). The CPP system was validated using a positive control (methamphetamine, 1 mg/kg, i.p.) in a different group of mice before the test with flubromazepam.

SA test

To elicit an operant response, animals were trained to press a lever to gain food pellets (Bio-Serv, Frenchtown, NJ, United States) in daily 3-h sessions until the desired target had been achieved (100 food pellets over 3 consecutive days). Animals that successfully achieved this target were selected. The animals ($n = 6$) were anesthetized with pentobarbital sodium (50 mg/kg; Entobar, Hanlim Pharm Co., Ltd, Seoul, Korea) and a catheter (0.3-mm inner diameter, 0.64-mm outer diameter; Dow Corning, Midland, TX, United States) was surgically inserted into right jugular vein of each mouse and made to exit at the right shoulder. Catheters were flushed with 0.2 mL of gentamicin sulfate (0.32 mg/mL; Shin Poong Pharm Co., Ltd, Seoul, Korea) in heparinized saline (30 IU/mL). The mice then received an intramuscular injection of penicillin (20,000 IU/mL) in saline, followed by 0.2 mL of heparinized saline every day during the experimental period. After the surgery, each animal was allowed to recover in a controlled cage for at least 7 days. The animals were treated with either the test compound or vehicle (DMSO:Tween 80:saline = 1:1:18, 0.1 mL/infusion) for 5 s during a 2 h session on a fixed-ratio 1 reinforcement schedule, meaning that if the animal pushed the active lever once, then the substance linked to the catheter was injected. The time-out period was set at 20 s. The SA test system was validated using methamphetamine (0.1 mg/kg/infusion, intravenous [i.v.]) prior to the test with flubromazepam.

Cytotoxicity in cardiomyocytes

H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA, United States) supplemented with 10% newborn calf serum (Gibco) and 1% antibiotics/antimycotics (Invitrogen, Carlsbad, CA, United States). Cells were incubated using standard culture methods at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. After reaching 70% confluence, H9c2 cells were counted and seeded in 96-well plates (100 μL/well, 1×10^4 cells per well). Following overnight incubation, the medium was replaced with fresh medium containing either solvent (DMSO:Tween 80:saline = 1:1:18) or varying concentrations of flubromazepam (0.1–100 μM). The final concentration of the solvent did not exceed 0.5% (v/v), which was determined to be nontoxic to the cell line. Cell viability was measured

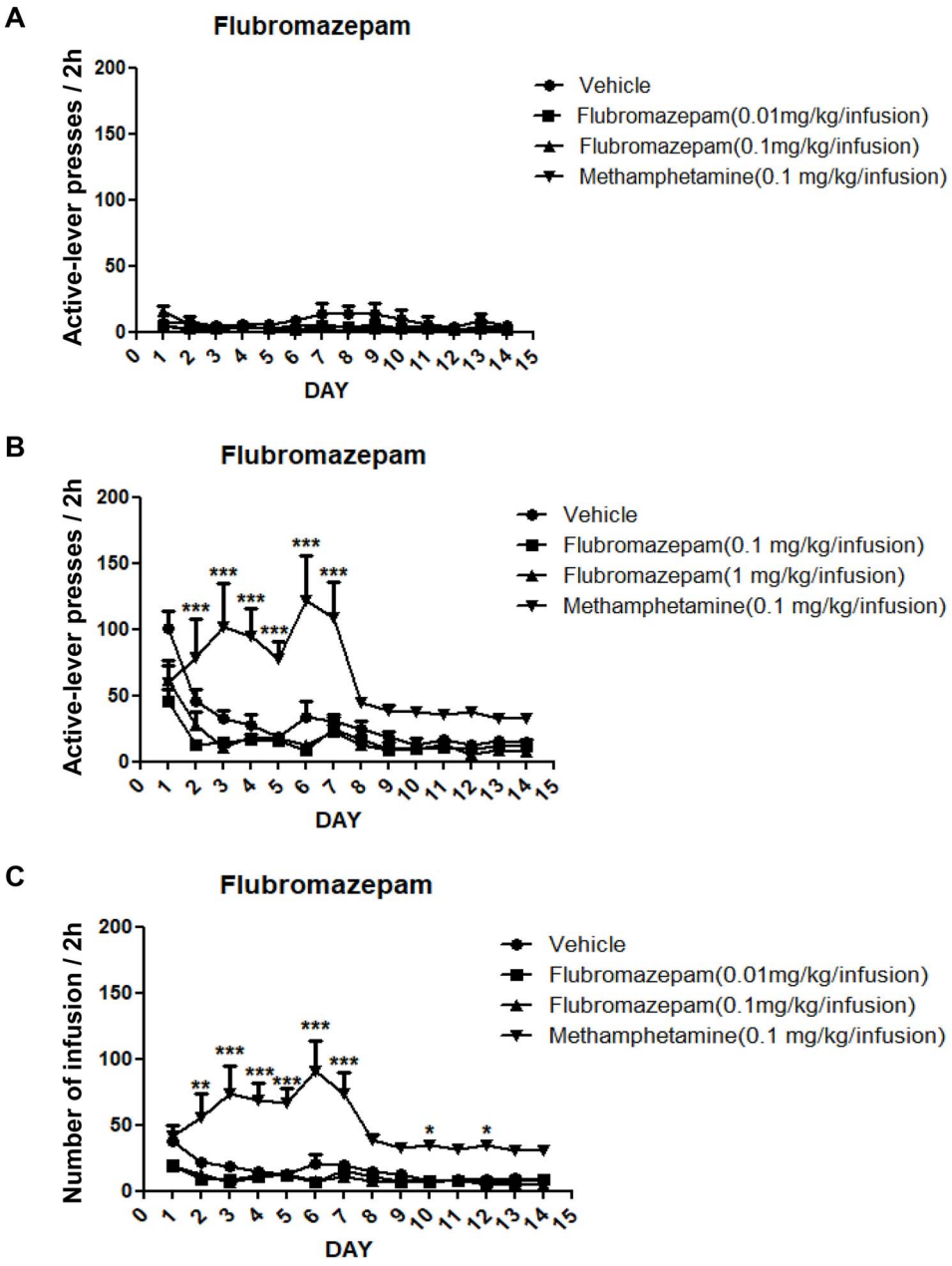

Fig. 3. Comparison of methamphetamine and flubromazepam in the SA behavioral paradigm. A) Inactive-lever presses, B) active-lever responses, and C) number of infusion. Data are expressed as the mean ± standard error of the mean of 6 animals per group. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistical significance compared with the vehicle control-treated group as determined by 2-way ANOVA followed by Bonferroni's post-hoc test.

using the MTT assay with the Cell Proliferation Kit I (Roche, Basel, Switzerland).[20] The optical density was measured at a wavelength of 570 nm using a microplate reader (SpectraMAX M5, Molecular Devices, Sunnyvale, CA, United States). Cell viability was calculated as a percentage of the measured optical density relative to that of the control group.

QT interval measurement by ECG of SD rats

To investigate the effects of flubromazepam on cardiac rhythm, ECG measurements were performed on rats. Male SD rats were injected with sodium pentobarbital (50 mg/kg, i.p.), followed by the administration of 1% isoflurane (100% oxygen) by inhalation to maintain anesthesia for >1 h. The anesthetized rats were restrained in a supine position. The body temperature of each animal was maintained by a rubber water bag filled with warm water. After shaving the chest of rat using electric clippers, an electrocardiogram was recorded in a noninvasive manner with needle electrodes placed at 3 optimized positions (left upper thorax, right upper thorax, and left lower thorax) in the skin of the rat to obtain the maximal amplitude recording for accurate measurement of the QT interval. ECG signals were recorded using an Animal Bio Amp and PowerLab 8/35. LabChart software (ADInstruments, Colorado Springs, CO, United States) was used to analyze the recorded ECG signals, including the QRS complex, isoelectric

level, P-wave, and T-wave, as well as to calculate the QT intervals. After measuring the basal level (10-min before administering the test compound), vehicle control (DMSO:Tween 80:saline = 1:1:18) or flubromazepam (0.8 and 2.0 mg/kg) was administered intravenously to the rats at an injection rate of 1 $mL \bullet kg^{-1} \bullet min^{-1}$ manually without a catheter. During ECG measurement, the total blood volume of the rats was calculated as 0.06 × body weight + 0.77 mL[21]; this was used to determine the injection concentration. QT intervals averaged each minute were corrected using the Bazett's equation ($QTc = QT/(RR)1/2$). The delta corrected QT interval (ΔQTc) was then derived using the basal level of the QT interval (QTcbasal, an average of the continuous QT interval for 10 min prior to drug administration) and the QT interval (QTceach) with the following equation: $\Delta QTc = QTceach - QTcbasal$.

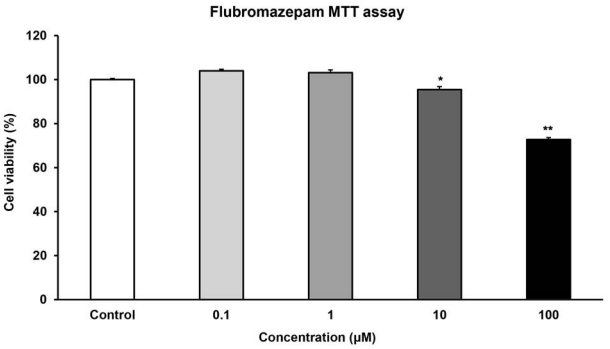

Fig. 4. Effects of flubromazepam on the viability of H9c2 cells. Cells were exposed to the test compound at indicated concentrations for 24 h, and cell viability was measured using the MTT assay. Data are presented as the mean ± standard error of the mean of 6 animals. Cell viability is expressed as a percentage relative to the control. $^{**}P < 0.01$ indicates statistical significance compared with the control group as determined by 1-way ANOVA followed by Bonferroni's post-hoc test.

Recording of hERG currents

To assess potassium channel inhibition by flubromazepam, the hERG assay was performed using Chinese hamster ovary (CHO) cells. CHO cells were cultured in 90% Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F12, Gibco), 9% fetal bovine serum (WelGENE Inc.), 0.9% penicillin/streptomycin (Gibco), and 50 μg/mL hygromycin B (Gibco). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for at least 7 days. Normal Tyrode's solution contained 143.0-mM NaCl, 5.4-mM KCl, 5.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.33-mM NaH_2PO_4, and 0.5-mM magnesium chloride ($MgCl_2$, pH 7.36–7.45), whereas the internal solution contained 130.0-mM KCl, 1.0-mM $MgCl_2$, 5.0-mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) (EGTA), 10.0-mM HEPES, and 5.0-mM magnesium salt of adenosine 5-triphosphate (Mg-ATP) (pH 7.27). Ionic currents were recorded in whole-cell voltage clamp mode using an Axopatch 200 B amplifier (Molecular Devices, Sunnyvale, CA, United States). After current amplification and digitization were performed using an Axopatch 200 B amplifier, the data were analyzed using Notocord software (Version 4.2, NOTOCORD INC., France). All tests were performed using a dual automatic temperature controller set to 36.9–37.1°C. To activate tail currents, cells were held at −80 mV and hyperpolarized at −90 mV for 100 ms. Cells were then depolarized to +20 mV for 2 s, followed by repolarization at −40 mV for 3 s. Next, the voltage pulse was activated consecutively for 20 s with the stimulation frequency set to 0.05 Hz. The concentration of the drug that inhibited ionic currents by 50% was calculated using the Hill equation: $f = XH/(IC50H + XH)$, where H is the Hill coefficient, IC50 is the 50% inhibitory concentration, X is the concentration, and f is the inhibition ratio.[22–25]

PAK1 protein levels in cardiomyocytes

The H9c2 cell line was maintained in DMEM containing 10% newborn calf serum and 1% antibiotics/antimycotics in a humidified atmosphere containing 5% CO_2. Cells were seeded in 6-well plates (1.0×10^6 cells per well) and incubated overnight. The culture medium was replaced with fresh medium containing either the vehicle solution (DMSO:Tween 80:saline = 1:1:18) or 100-μM flubromazepam. The final concentration of the vehicle did not exceed 0.25% (v/v), which was determined to be nontoxic to the cell line. After incubation, H9c2 cells were lysed at 4°C in a radioimmunoprecipitation assay buffer containing protease inhibitors (Thermo Fisher Scientific, Waltham, MA, United States). Cell lysates were used for western blot experiments. Proteins extracted from treated H9c2 cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%; Bio-Rad, Hercules, CA, United States). The separated proteins were transferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA, United States). After blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 at room temperature (23–25°C) for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against PAK1 (rabbit, 1:250, Sigma–Aldrich). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1,000, Sigma–Aldrich) at room temperature for 1 h. The signal for PAK1 protein was detected using enhanced chemiluminescence with an ECL Plus detection system (GE Healthcare, Piscataway, NJ, United States) and a ChemiDoc MP imaging system (Bio-Rad). Images were captured with Image Lab 4.1 gel documentation software (Bio-Rad). Rat primary cardiomyocytes were obtained using a Pierce primary cardiomyocyte isolation kit (Thermo Fisher Scientific) and maintained in DMEM with 10% HI FBS and 1% antibiotics/antimycotics in an atmosphere containing 5% CO_2. The cells were plated in a 6-well plate (2.5×10^6 cells per well) and incubated overnight. The medium was replaced according to the manufacturer's instructions for 7–10 days, and the cells were treated with fresh medium containing 100-μM solvent (DMSO:Tween 80:saline, 1:1:18) or 100-μM flubromazepam after full

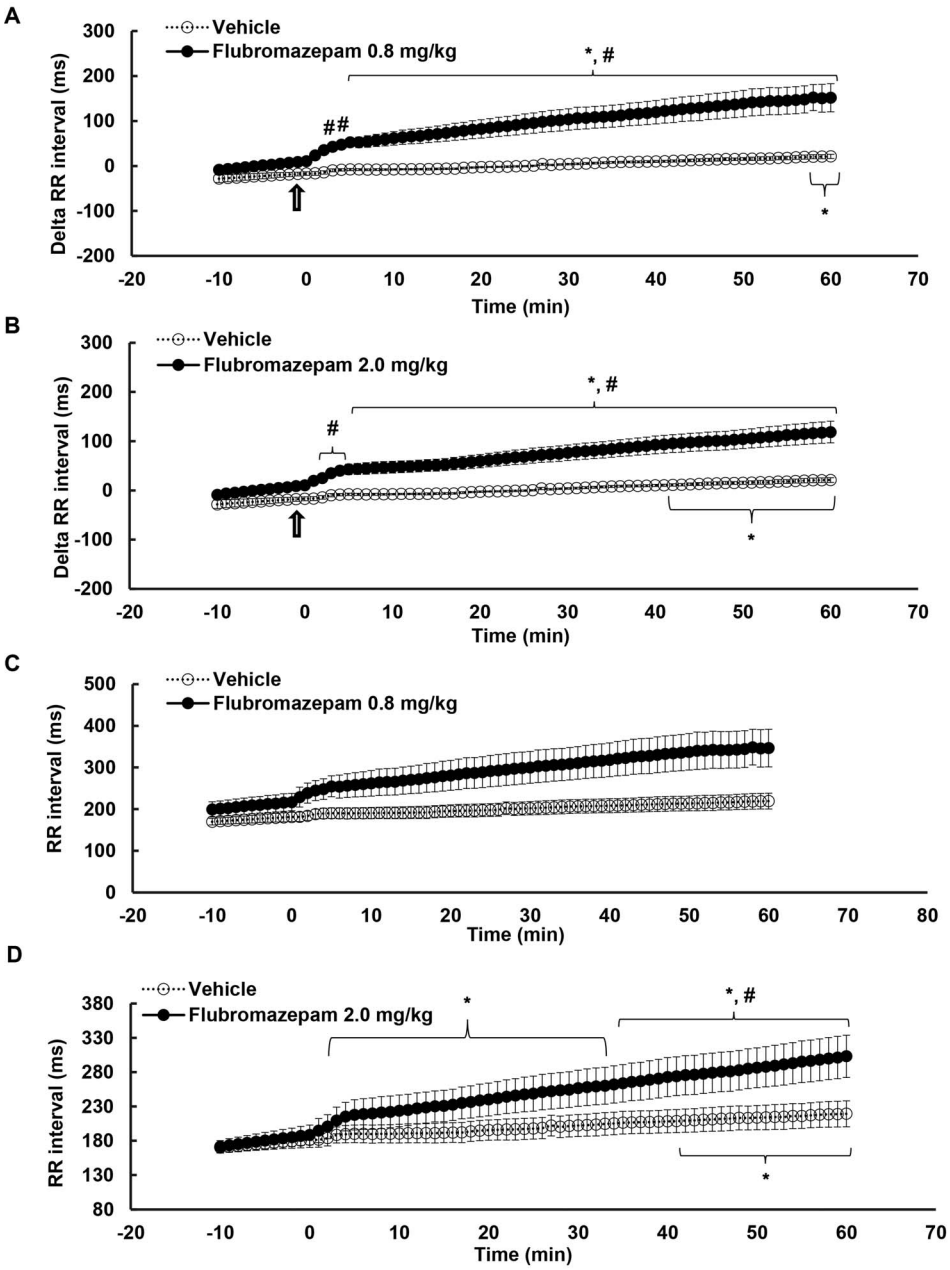

Fig. 5. Effects of flubromazepam on delta RR intervals A, B) and RR intervals C, D) in the hearts of Sprague Dawley rats. Electrocardiograms of animals treated with vehicle or 2 doses of flubromazepam (0.8 mg/kg A, C); 2 mg/kg B, D)) were recorded before and after injection. Representative electrocardiogram recordings show electrocardiographic patterns and variations between the vehicle control group and flubromazepam-treated group.

culture. Western blotting analysis was carried out using the same method used for the H9c2 cells.

Statistical analysis

SigmaStat (https://systatsoftware.com/) was used to analyze the in vitro and in vivo results. All data are presented as mean ± standard error of the mean. Statistically significant differences between the vehicle- and flubromazepam-treated groups were analyzed using Student's *t*-tests. One-way repeated measures analysis of variance (RM ANOVA), 2-way RM ANOVA, and Bonferroni's tests were used for equal variance data, while Dunnett's test was used for non-equal variance data. Statistical significance was set at $P < 0.05$.

Results

CPP testing indicates flubromazepam dependence in mice

CPP testing was performed to determine the dependence potential of flubromazepam. Prior to testing flubromazepam, the test system was validated using a positive control (methamphetamine, 1 mg/kg, i.p.; Fig. 2A). After validation, flubromazepam (0.01 and 0.1 mg/kg, i.p.) was administered to the experimental animals, and the CPP

test was conducted using mice that exhibited no initial preference for any specific compartment. Following a conditioning phase with flubromazepam, preference for the drug-paired compartment was determined by comparing the time spent in the drug-paired compartment between the preconditioning and post-conditioning phases. We discovered that 0.1 mg/kg flubromazepam considerably increased the time spent in the drug-paired compartment (Fig. 2B).

SA testing does not indicate flubromazepam dependence in mice

SA tests were conducted as an alternative method for assessing the dependence potential of flubromazepam. We validated the experimental design by using methamphetamine as a positive control (0.1 mg/kg/infusion, i.v.), which considerably increased the active-lever responses and number of infusions (Fig. 3A and B), but not the inactive-lever responses (Fig. 3C), of mice. To determine the doses of flubromazepam, we considered the experiences of drug abusers who reported flubromazepam use up to 16 mg/60 kg of body weight.[26] We calculated the corresponding dose for mice to be 3.2 mg/kg using an established translational equation.[1] In addition, we performed a pilot study using climbing behavior tests in which we tested flubromazepam doses of 0.01, 0.1, 0.5, and 10 mg/kg and found that neither 0.01 nor 0.1 mg/kg flubromazepam had an adverse effect on motor function; therefore, we selected these doses for use in SA testing. No significant behavioral changes were observed in the flubromazepam-treated groups (Fig. 3A–C).

Flubromazepam impairs cardiomyocyte viability

To examine the effect of flubromazepam on cardiomyocyte viability, H9c2 cells were treated with 4 concentrations (0, 0.1, 1, 10, or 100 μM) of flubromazepam for 24 h and subsequently subjected to MTT assays. Following treatment with 100-μM flubromazepam, the viability of H9c2 cells was reduced by >20% compared with vehicle-treated controls. Therefore, we identified 100 μM as the toxic concentration of flubromazepam in H9c2 cells (Fig. 4).

Flubromazepam affects heart function

To determine the effect of flubromazepam on cardiac rhythm, ECGs were performed for confirmation of QT and RR intervals on SD rats following administration of 0.8 or 2 mg/kg flubromazepam. The QT and RR intervals are the factors on the electrical properties of the heart, and the corrected QT interval (QTc) is the calculated correction value for the effect of heart rate (RR interval root) on the measured QT interval. Flubromazepam treatment did not alter QTc intervals (data not shown). Both doses of flubromazepam markedly increased delta RR intervals compared with data recorded 1-min prior to drug administration as well as the vehicle control group (Fig. 5).

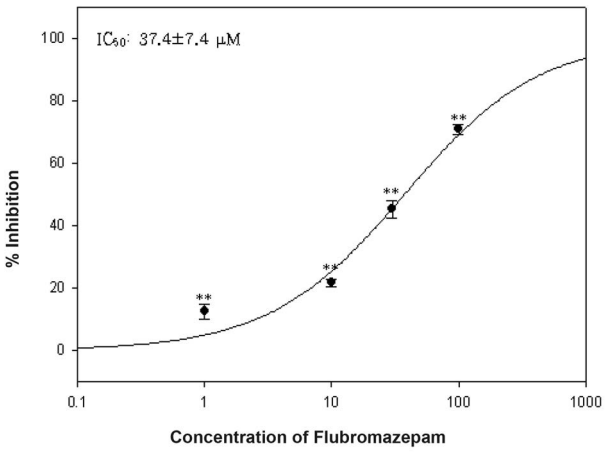

Fig. 6. Effects of flubromazepam on the hERG potassium channel. Flubromazepam inhibited hERG potassium channels in a concentration-dependent manner. Data points are presented as means ± standard error of the mean. $^{**}P < 0.01$ indicates statistical significance compared with the control group as determined by 1-way ANOVA followed by Dunnett's post-hoc test.

Flubromazepam inhibits potassium channels

As flubromazepam induced arrhythmia, its effect on potassium channels was examined in CHO cells using the hERG assay. Flubromazepam inhibited hERG potassium channels in a concentration-dependent manner, with an IC50 value of 37.4 ± 7.4 μM (Fig. 6).

Flubromazepam does not affect PAK1 expression

PAK1 is a known biomarker of cardiotoxicity. To investigate the relationship between cardiac effects and PAK1 expression, PAK1 protein levels were analyzed in H9c2 cells or rat primary cardiomyocytes following treatment with flubromazepam using western blotting. We found that 100-μM flubromazepam did not alter PAK1 protein expression compared with vehicle-treated control cells (Fig. 7A and B).

Discussion

NPS abuse is an emerging issue in global. For appropriate regulation against designer drugs, pharmacological and toxicological data are required. Among toxicity, especially dependence potential and cardiovascular toxicity should be evaluated in in vivo and in vitro models. According to United Nations Office on Drugs and Crime (UNODC), benzodiazepine-type substances are a primary NPS, identified in ~70% of toxicological cases. Flubromazepam is a 1,4-Benzodiazpepine based chemical which class is most abused among benzodiazepines.[27] Benzodiazpines are considered to have an agonistic activity on GABAA receptor for their psychoactive properities. However, as a designer benzodiazepine, flubromazepam has been the subject of investigation in recent years; however, there has been no reliable data reported on its adverse effects.[28] Benzodiazepines induce disinhibition of GABAergic inhibitory regulation on mesolimbic dopamine neuronal

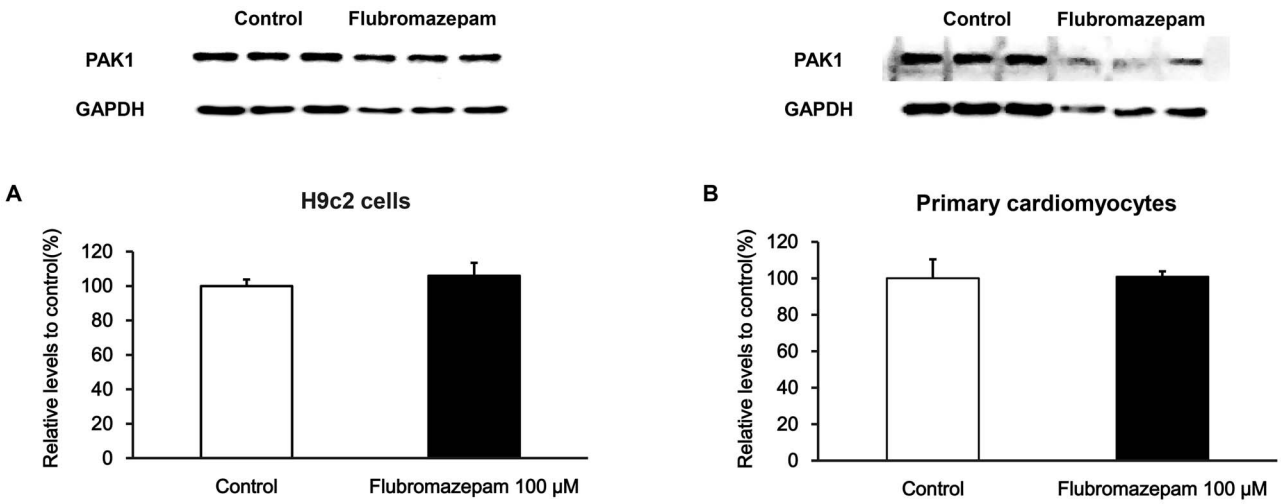

Fig. 7. Effects of flubromazepam on PAK1 in H9c2 cells A) and primary cardiomyocytes B). Data are presented as means ± standard error of the mean of 4 replicates. **$P < 0.05$ indicates statistical significance compared with the control group as determined by 1-way ANOVA followed by Dunnett's post-hoc test.

activity[29]; therefore, flubromazepam may also regulate dopaminergic neuronal function in the reward pathway in the brain. In this study, we demonstrated that flubromazepam induced reward-related behavior in CPP tests, but not in SA tests. It should be noted that the degree of the reward-enhancement induced by 0.1 mg/kg of flubromazepam was comparable with that of 1 mg/kg of methamphetamine, a well-known "hard drug." In contrast to CPP tests, SA tests are operant behavior tests that require SA of the reward to detect drug-induced reinforcement.[30] We hypothesize that the structure of flubromazepam may explain the conflicting results obtained from the CPP and SA tests. Flubromazepam contains halogen elements in its structure, such as fluorine (F) and bromine (Br), which may increase lipophilicity. The high lipophilic properties of drugs may contribute to their capacity for blood–brain barrier penetration. In addition, high lipophilicity increases drug distribution to adipose tissues, which can result in delayed release. Flubromazepam has a prolonged half-life of 106 h[28] and, based on the experiences of drug abusers, the onset of effects of flubromazepam occurs 40–90 min after administration.[26]However, we did not observe any motor impairment at a dose of 0.1-mg/kg flubromazepam (data not shown). Therefore, we suggest that the pharmacokinetic properties of the drug underly the conflicting CPP and SA behaviors following administration of flubromazepam.

Several studies have reported that both flubromazepam and flubromazolam, a triazole analogue of flubromazepam, induce cardiovascular symptoms in humans, such as hypertension, tachycardia, and bradycardia[15,16,31,32]; however, the molecular mechanisms associated with the reported cardiovascular effects of flubromazepam are not well understood. We have been studying cardiotoxicity induced by various drugs, including NPSs, and have reported that PAK1 is a useful biomarker for cardiotoxicity caused by NPSs.[33–37] However, although flubromazepam induced cytotoxicity in cardiomyocytes at a range of doses, PAK1 was not altered by flubromazepam, even at the highest dose tested (100 μM; data not shown).

hERG channels play an important role in repolarization of the heart, and their inhibition induced by cardiotoxic drugs is associated with arrhythmia.[38] hERG inhibition and the associated prolongation of the QT interval are severe cardiac effects caused by abnormal myocardial repolarization that are associated with the risk of ventricular tachycardia, ventricular fibrillation, and life-threatening arrhythmia.[39–41] Although QTc intervals were unaffected in flubromazepam-treated animals, delta RR intervals considerably increased, indicating that flubromazepam may induce bradycardia. In agreement with our findings, benzodiazepines have been reported to inhibit hERG channels in vitro but have not been found to alter QT duration in clinical use,[42–44] and other studies have reported bradycardia as an adverse effect of other benzodiazepines.[45,46] Thus, these results suggest that delta RR interval prolongation may play a role in the bradycardiac effects of designer benzodiazepines in humans.

Conclusions

The present study is the first to evaluate the dependence potential and cardiotoxicity of flubromazepam in vivo and in vitro. Flubromazepam treatment induced reward-related behavior in CPP tests and abnormal ECGs. The use of animal studies for obtaining data on drug effects is useful for developing regulatory policies against NPSs, which often lack pharmacological and toxicological data. Our results suggest that flubromazepam may be harmful to human health. Additional data on the effects of flubromazepam on the central nervous system and other systems in the body are required to gain a better understanding of the need to control this compound as a

psychotropic substance at the national and international levels.

Author contributions

H.J.C. and J.Y. conceptualized and designed the study; S.M.G., J.M.K. and K.S.Y. were involved in data curation; E.H., S.M.G. and J.Y. were involved in formal analysis; J.Y. took responsibility of funding acquisition; E.H., S.M.G., J.-M.L., Y.-H.K., and S.K.S. did investigation; H.J.C. and J.Y. were involved in study design methodological development; Y.-H.K. and S.K.S. were involved in project administration; H.J.C. and J.Y. have responsibility of resources; J.M.K., K.S.Y., J.-M.L., and S.K.S. were involved in software development; H.J.C. and J.Y. did supervision; E.H and J.Y. were involved in visualization; E.H. and J.Y. led the drafting of the manuscript with input from all authors; D.L., S.M.G., H.E., H.J.C. and J.Y were involved in writing – revision and editing and have responsibility for its final content. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by the Ministry of Food and Drug Safety (19182MFDS415, 20182MFDS422, 20182MFDS425), the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. MRC, 2017R1A5A2015541), and "Regional Innovation Strategy (RIS)" through the NRF funded by the Ministry of Education (MOE) (2021RIS-001).

Conflicts of interest. There are no conflicts of interest to be declared.

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